Published in final edited form as:

Brain Res. 2011 August 17; 1407: 107–122. doi:10.1016/j.brainres.2011.06.042.

Serine proteases, serine protease inhibitors, and protease-activated receptors: roles in synaptic function and behavior

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Abstract

Serine proteases, serine protease inhibitors, and protease-activated receptors have been intensively investigated in the periphery and their roles in a wide range of processes—coagulation, inflammation, and digestion, for example—have been well characterized (see Coughlin, 2000; Macfarlane et al., 2001; Molinari et al., 2003; Wang et al., 2008; Di Cera, 2009 for reviews). A growing number of studies demonstrate that these protein systems are widely expressed in many cell types and regions in mammalian brains. Accumulating lines of evidence suggest that the brain has co-opted the activities of these interesting proteins to regulate various processes underlying synaptic activity and behavior. In this review, we discuss emerging roles for serine proteases in the regulation of mechanisms underlying synaptic plasticity and memory formation.

Keywords

Protease-activated receptor; protease; serpin; hippocampus; plasticity

1. Introduction

Serine proteases, serine protease inhibitors, and protease-activated receptors (PARs) have classically been studied for their effects in coagulation, hemostasis and hemodynamics, inflammation, and wound healing (Cirino et al., 1996; Leger et al., 2006; Kaneider et al., 2007; Niessen et al., 2008; also see Coughlin, 2000; Macfarlane et al., 2001; Molinari et al., 2003; Wang et al., 2008; and Di Cera, 2009 for reviews). Over the past decade, it has become increasingly apparent that these proteins are found endogenously in the central nervous system (CNS), suggesting roles for these proteins in normal physiology and disease states in this tissue. Various groups have thus shown that serine proteases, their zymogen precursors and endogenous inhibitors, and PAR substrates can influence synaptic function and behavior. Furthermore, aberrant activity of these molecules make important contributions to a number of neurological disorders such as Alzheimer's disease, Parkinson's disease, traumatic brain injury, and stroke (Akiyama et al., 1992; Cinelli et al., 2001; Junge et al., 2003; Olson et al., 2004; Nicole et al., 2005; Hamill et al., 2007; Neilsen et al., 2007; Fabbro and Seeds, 2009).

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There are several objectives for this review. First, we will discuss serine proteases and serine protease inhibitors that are endogenously expressed in the brain and have been shown to have effects on synaptic function, particularly in the context of long-term plasticity, and in behavior and cognition. Second, we will discuss protease-activated receptors, a family of intriguing G-protein coupled receptors (GPCRs) that are targets for various proteases, and their emerging roles in modulating synaptic plasticity and behavior. Finally, we will examine mounting evidence that PARs participate in astrocyte-neuron interactions which may underlie behavior and synaptic plasticity. Throughout this review, the updated nomenclature for ionotropic glutamate receptor subunits will be used (Collingridge et al., 2009).

2. Serine proteases

Serine proteases are a diverse group of enzymes that are characterized by the presence of three critical amino acids—histidine, aspartate, and serine—in the catalytic site (Davies et al., 1998; Di Cera, 2009). Indeed, the “serine” in the name serine proteases refers to the involvement of the catalytic site serine in the catalysis of proteolysis in this category of enzymes. A class of serine proteases, the thrombin-like proteases, which include thrombin, tissue plasminogen activator (tPA), and plasmin, are best known for their roles in coagulation and inflammation responses (as reviewed in Coughlin, 2000). Another class of serine proteases, the trypsin-like proteases, which include trypsin, neurotrypsin, and neuropsin, were first characterized for their functions in food digestion (as discussed in Luo et al., 2007; Wang et al., 2008). We now know that many serine proteases are expressed in the brain and play roles in development, maintenance, and pathology of the nervous system (see Turgeon and Houenou, 1997, Davies et al., 1998, Gingrich and Traynelis, 2000; Luo et al., 2007; and Wang et al., 2008 for more detailed discussions). The actions of serine proteases are regulated by another class of proteins, the *serine protease inhibitors*, or serpins (Miranda and Lomas, 2006). Burgeoning evidence suggests that these molecules also contribute to brain physiology and pathophysiology (as discussed in Tomimatsu, et al., 2002; Miranda and Lomas, 2006; and Wang et al., 2008). The following section will highlight the roles of several serine proteases and serpins in regulating synaptic function and behavior.

2.1. Thrombin

Thrombin is well known for its actions in regulating platelet aggregation, endothelial cell activation, and fibrinolysis (Sweatt et al., 1985; Hung et al., 1992; Coughlin, 2000). Although thrombin can enter the brain in pathological situations in which the blood-brain barrier is compromised (Gingrich and Traynelis, 2000; Junge et al., 2003; Xi et al., 2003), thrombin and its precursor, prothrombin, are synthesized in the brain, suggesting roles in normal brain function (as reviewed in Turgeon and Houenou 1997; and Turgeon et al., 2000). Thrombin has been shown to mediate a host of effects on both neurons and glial cells. Application of thrombin to cultured neurons and neuroblastoma cells inhibits neurite outgrowth (Gurwitz and Cunningham, 1988; Jalink and Moolenaar, 1992; Suidan et al., 1992; Gill et al., 1998) by stimulating collapse of the growth cone (de la Houssaye et al., 1999). Astrocytes also display a number of responses to thrombin. Thrombin induces reversal of stellate morphology in cultured astrocytes, as well as stimulating proliferation (Rohatgi et al., 2004b). In addition, treatments with high concentrations of thrombin induce apoptosis in both neurons and astrocytes (Donovan et al., 1997). These and other observations suggest that thrombin signaling may be important for neuronal development and maintenance (for further discussions, see Turgeon and Houenou, 1997; Turgeon et al., 2000; and Rohatgi et al., 2004b).

Observations that thrombin entry into the brain, such as following an intracerebral hemorrhage, can lead to the generation of seizures suggest that thrombin signaling may influence neuronal activity in the fully mature nervous system as well (Lee et al., 1997; Xi et al., 2003). Indeed, thrombin has been demonstrated to have effects on synaptic function. When applied to hippocampal slices, thrombin potentiates N-methyl-D-aspartate receptor-(NMDAR) mediated currents (Gingrich et al., 2000). While high concentrations of thrombin can cleave GluN1 subunits, this potentiation of NMDAR-mediated function is independent of thrombin's proteolytic actions. In another study, a short thrombin exposure induced a slow, long-lasting enhancement of field excitatory post-synaptic potentials (fEPSPs) recorded in area CA1 that eventually resulted in long-term potentiation (LTP; Maggio et al., 2008). This LTP was saturated, as tetanic stimulation after thrombin application did not result in further potentiation. Furthermore, this thrombin-induced LTP was NMDAR-dependent, as it was blocked by the NMDAR antagonist APV. Altogether, these observations suggest that thrombin signaling in the brain converges on NMDAR function to regulate synaptic activity and long-term plasticity.

High levels of thrombin in the brain have also been associated with deleterious effects on cognitive function. Rats treated with intracerebroventricular infusion of thrombin committed significantly more reference memory errors and had higher task completion latencies in the eight-arm radial maze (Mhatre et al., 2004). Concomitant with these behavior deficits were a number of neuropathological changes, including enlarged cerebral ventricles, increased astrogliosis, and increased cell death. This study also found increased levels of phosphorylated neurofilament proteins and increased levels of apolipoprotein E (ApoE). This observation is consistent with other reports which suggest that thrombin may have a role in regulating some aspects of Alzheimer's disease neuropathology and cognitive decline (see Akiyama et al., 1992; Turgeon and Houenou, 1997; Turgeon et al., 2000; Mhatre et al., 2004; and Rohatgi et al., 2004b for more detailed discussions).

2.2. Tissue plasminogen activator

Tissue plasminogen activator (tPA), the standard of care for acute ischemic stroke, is the most intensively studied serine protease in the CNS. In the brain, tPA is synthesized and released by neurons, glial cells, and endothelial cells, and it is highly expressed in various brain regions, including the cerebellum, cortex, amygdala, and hippocampus (Qian et al., 1993; Tsirka et al., 1995; Sallés and Strickland, 2002; Shin et al., 2004; Yepes and Lawrence, 2004a; Yepes and Lawrence, 2004b; Lochner et al., 2006). A number of studies have implicated tPA as a modulator of synaptic activity, although there is some debate surrounding the underlying mechanisms (see Samson and Medcalf, 2006). On one hand, the proteolytic activity of tPA could enhance NMDAR signaling through cleavage of the GluN1 subunit (Nicole et al., 2001; Kvajo et al., 2004; Benchenane et al., 2007; but also see Matys and Strickland, 2003; Samson et al., 2008). It is thought that this cleavage enhances calcium permeability through the NMDAR, as evidenced by an NMDAR-dependent rise in intracellular calcium that ultimately results in increased neuronal damage in an *in vitro* model of excitotoxicity. On the other hand, tPA could be interacting with the NMDAR in a non-proteolytic fashion. On GluN2B-containing NMDARs, tPA can interact with the GluN2B subunit and facilitate phosphorylation of GluN2B, which leads to activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, and results in further upregulation of GluN2B-containing NMDARs (Pawlak et al., 2005a; Norris and Strickland, 2007). Alternatively, tPA could potentially indirectly influence NMDAR function by mediating an interaction requiring engagement of a low-density lipoprotein receptor (Bacskai et al., 2000; Qiu et al., 2002; Yepes et al., 2003; Samson and Medcalf, 2006; Benarroch, 2007; and Samson et al., 2008).

These reported interactions between tPA and NMDAR subunits suggest that tPA activity may affect synaptic plasticity. Indeed, in pioneering LTP experiments in hippocampal slices, inhibition of tPA activity or deletion of the tPA gene inhibited late-phase LTP (Frey et al., 1996; Huang et al., 1996; Baranes et al., 1998; Zhuo et al., 2000). Moreover, tPA $-/-$ mice also have impaired long-term depression (LTD) in striatal slices (Calabresi et al., 2000). Interestingly, hippocampal slices from transgenic mice overexpressing tPA in neurons displayed increases in both paired-pulse facilitation and LTP magnitudes compared to slices from wild-type mice, providing further evidence that tPA activity can modulate synaptic function (Madani et al., 1999).

These slice electrophysiology results are further supported and extended by observations that tPA activity can influence behavior. tPA knockout mice display impairments in multiple learning and memory tasks, including altered escape latencies in active and step-down avoidance tests, impaired reactivity to spatial and object novelty, lower freezing levels in contextual fear conditioning, and impaired acquisition of a cerebellum-dependent motor learning task (Huang et al., 1996; Pawlak et al., 2002; Calabresi et al., 2000; Seeds et al., 2003; Benchenane et al., 2007).

tPA activity also seems to play a critical role in mediating various responses to stress. In a model of acute restraint stress, upregulation of tPA activity in the amygdala preceded the onset of stress-induced increased anxiety-like behavior in the elevated-plus maze in wild-type mice (Pawlak et al., 2003). In the hippocampus, tPA knockout mice subjected to acute restraint stress did not show tPA-dependent increases in GluN2B expression and extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation that were observed in wild-type mice, and stressed tPA knockout mice showed less freezing behavior than stressed wild-type mice in contextual fear conditioning (Norris and Strickland, 2007). Wild-type and tPA knockout mice subjected to a model of chronic stress revealed a few striking tPA-dependent effects in the hippocampus (Pawlak et al., 2005b). First, there were large stress-induced decreases in GluN1, GluN2A, and GluN2B expression levels in wild-type mice, with hippocampal area CA1 showing the most pronounced decreases. Second, chronic stress induced a significant decrease in spine density on CA1 pyramidal cells. Lastly, these decreases in NMDAR subunit levels and spine density were associated with impaired learning in the Morris water maze. In contrast, tPA knockout mice showed blunted decreases in NMDAR subunits after chronic stress, no changes in spine density, and no spatial learning deficits (Pawlak et al., 2005b).

While it is clear that tPA plays an important role in normal brain function, its effects are likely not direct. tPA is a narrow spectrum protease, and its primary substrate, plasminogen, is also normally found in the brain.

2.3. Plasmin

Plasminogen is cleaved by tPA to the broad specificity protease plasmin (as reviewed in Yepes and Lawrence, 2004a; and Yepes and Lawrence, 2004b). Plasmin has also been demonstrated to influence brain function, presumably through its proteolytic activity. Like tPA, plasminogen application causes an NMDAR-mediated rise in intracellular calcium concentrations in neurons (Inoue et al., 1994). Plasmin activity has also been shown to be important for some forms of LTP. In one study, simultaneous application of plasminogen or plasmin and a subthreshold tetanus facilitated the induction of LTP (Mizutani et al., 1996). In addition, plasmin cleavage of the precursor pro-brain-derived neurotrophic factor (proBDNF) to mature BDNF is critical for expression of late-phase LTP (Pang et al., 2004). Interestingly, plasmin activation of BDNF in the suprachiasmatic nucleus can modulate glutamate-induced phase shifts, indicating a role for plasmin in synaptic plasticity underlying circadian rhythms (Mou et al., 2009). More recently, it has been found that

plasmin can cleave the amino-terminal domain (ATD) of the GluN2A subunit of the NMDAR (Yuan et al., 2009). This cleavage is thought to remove the high affinity zinc binding site within the ATD, thereby relieving zinc inhibition of NMDAR function which could, in turn, affect LTP. In contrast to these effects, one study suggests that plasmin could negatively regulate LTP (Nakagami et al., 2000). In this study, organotypic hippocampal slice cultures incubated with plasmin exhibited impaired LTP maintenance, which was attributed to plasmin degradation of a laminin-mediated cell-extracellular matrix interaction (Nakagami et al., 2000). At the whole animal level, behavior studies have shown that microinjection of plasmin in the nucleus accumbens can potentiate morphine-induced dopamine release and hyperlocomotion in mice, suggesting a role for plasmin in addiction (Nagai et al., 2005; Nagai et al., 2008).

While the extent of plasmin's proteolytic substrates and downstream signaling in the brain are not well understood (Fig. 1), one particular substrate, PAR1, has emerged as an exciting target for further study.

2.4. Trypsin

Trypsin is well-known for its important roles in food digestion (Szmola et al., 2003; Wang et al., 2008; Koistinen et al., 2009). Trypsin is primarily produced by cleavage of the pancreatic proenzyme, trypsinogen, by enterokinases in the gastrointestinal tract (Luo et al., 2007; Wang et al., 2008; Koistinen et al., 2009). While eight different trypsin genes have been found in humans, only three code for protein: trypsinogen 1 (cationic trypsinogen), trypsinogen 2 (anionic trypsinogen), and trypsinogen 3 (mesotrypsinogen) (Luo et al., 2007; Wang et al., 2008; Koistinen et al., 2009). Additionally, an alternatively spliced form of mesotrypsin, trypsinogen 4, is the only trypsin isoform expressed in the human brain (Wiegand et al., 1993; Tóth et al., 2007). Trypsinogen 4 is widely distributed in the brain, and is expressed in both neurons and glia (Tóth et al., 2007). While physiological roles for this brain-specific trypsin are still unknown, emerging evidence suggests that it can activate several PARs (Cottrell et al., 2004; Grishina et al., 2005; Wang et al., 2006; Bushell, 2007; Knecht et al., 2007).

2.5. Neurotrypsin

Neurotrypsin, a novel trypsin-like, brain-specific serine protease was independently and concurrently identified by two laboratories in 1997 (Gschwend et al., 1997; Yamamura et al., 1997). In human and mouse brain, neurotrypsin is highly expressed in the hippocampus and amygdala, areas important in learning and memory (Gschwend et al., 1997; Molinari et al., 2002). Electron microscopy studies in human brain (Molinari et al., 2002) and confocal microscopy studies of cultured mouse hippocampal neurons transfected with EGFP (Frischknecht et al., 2008) indicate that neurotrypsin is localized to presynaptic terminals. Neurotrypsin is secreted from neurons in an activity-dependent manner, and remains at the synapse for several minutes (Frischknecht et al., 2008). One identified substrate for neurotrypsin is the extracellular proteoglycan, agrin (Reif et al., 2007; Stephan et al., 2008). Proteolytic cleavage of agrin has been proposed to regulate the organization and/or maintenance of synapses underlying synaptic plasticity (Mitsui et al., 2009; also see Molinari et al., 2003 and Wang et al., 2008 for reviews). In support of this idea, neurotrypsin knockout mice demonstrated abnormal social behavior and showed significantly decreased spine density in hippocampal neurons (Mitsui et al., 2009). Moreover, clinical studies reveal that aberrant neurotrypsin activity underlies a form of autosomal recessive mental retardation (Molinari et al., 2002; also see Molinari et al., 2003 for a review). A four-base pair deletion in the neurotrypsin gene results in a truncated protein lacking the serine protease catalytic domain. In individuals from two families in Algeria, this deletion resulted

in severe cognitive impairment by two years of age, despite normal psychomotor development during the first 18 months (Molinari et al., 2002; Molinari et al., 2003).

2.6. Neuropsin

Neuropsin is another trypsin-like serine protease which was initially found in the brain but has since been found in other organs (Chen et al., 1995; Wang et al., 2008). Like many of the serine proteases discussed thus far, neuropsin is highly expressed in the pyramidal cell layer in hippocampal areas CA1–3 and in the lateral amygdala (Chen et al., 1995; Attwood et al., 2011). While the localization of neuropsin would lead to the hypothesis that neuropsin activity could influence synaptic plasticity and behavior, experiments testing this hypothesis have shown inconsistent results. In hippocampal slice recordings, perfusion of low concentrations of neuropsin (1–2.5 nM) coupled with a theta-burst stimulation protocol to induce LTP resulted in a marked enhancement of early-phase LTP (Komai et al., 2000). Curiously, perfusion of varying concentrations to further characterize this effect showed a bell-shaped concentration-response curve, which exhibited peak LTP enhancement at ~2.5 nM but exhibited decreasing levels of potentiation with increasing neuropsin concentrations (Komai et al., 2000). A possible explanation for this effect comes from the observations that cell adhesion molecule L1 is a neuropsin substrate, and that neuropsin proteolysis of L1 coincided with NMDAR-dependent synaptic activity (Matsumoto-Miyai et al., 2003). This is consistent with a previous observation that inhibition of L1 cleavage resulted in reduced LTP magnitudes (Lüthi et al., 1994). Additionally, a recent study demonstrates that the EphB2 receptor is another neuropsin substrate, and that EphB2 cleavage by neuropsin regulates an EphB2-NMDAR interaction that controls processes underlying anxiety-related behavior and LTP in the amygdala (Attwood et al., 2011). Together, these observations suggest that neuropsin can modulate behavior and long-term synaptic plasticity through multiple proteolytic targets.

Neuropsin knockout mice have been generated by two groups using differing gene disruption strategies (Hirata et al., 2001; Davies et al., 2001). The mice generated by Davies et al. presented with normal hippocampal cytoarchitecture, normal input-output curves, normal short-term plasticity, unimpaired LTP, and normal spatial memory formation in the Morris water maze (Davies et al., 2001). These mice, however, did show hyperexcitability in the form of increased polyspiking in response to repetitive afferent stimulation, and showed heightened seizure activity in the kainate seizure model (Davies et al., 2001). In contrast, the mice generated by Hirata et al. displayed enlarged and elongated pyramidal cell bodies and a significant decrease in excitatory synapses in *stratum radiatum*, changes suggesting that behavior and synaptic function may be impaired (Hirata et al., 2001). Indeed, these mice showed impaired hippocampus-dependent learning in the Morris water maze and Y-maze tasks as well as impaired LTP in *in vivo* experiments (Tamura et al., 2006). Clarification of the basis of these differences in experimental outcome awaits further investigation.

3. Serine protease inhibitors

Serine protease inhibitors, or serpins, comprise a family of proteins that antagonize the activity of serine proteases. These proteins inhibit protease activity by a conserved mechanism involving a profound conformational change (as reviewed in Miranda and Lomas, 2006; Wang et al., 2008; and Ricagno et al., 2009). In this mechanism, the serpin presents a substrate-mimicking peptide sequence—the reactive center loop—to its target serine protease. Cleavage of the reactive center loop triggers a conformational change in which the bound protease translocates from the top to the bottom of the serpin molecule; simultaneously, part of the cleaved reactive center loop inserts into the β -sheet A of the serpin, thereby irreversibly inactivating the protease (Huntington et al., 2000; Briand et al., 2001).

As with serine proteases, there is a growing appreciation for the actions of serpins in normal and abnormal brain function. Table 1 lists serpins that have been demonstrated to inhibit the serine proteases previously discussed. A few recent reviews have examined the roles of these serpins in brain development, function and disease in detail (Yepes and Lawrence, 2004a; Yepes and Lawrence, 2004b; Miranda and Lomas, 2006; Wang et al., 2008). The following section will focus on two particular serpins, protease nexin-1 and neuroserpin, which have been found to have profound effects on synaptic plasticity and cognitive function.

3.1. Protease nexin-1

Protease nexin-1 (PN-1) was the first serpin identified in the brain (Wang et al., 2008). PN-1 is a potent inhibitor of thrombin but can also inhibit tPA, plasmin, and trypsin (Lüthi et al., 1997; Miranda and Lomas, 2006; Wang et al., 2008; Koistinen et al., 2009). In the CNS, PN-1 is synthesized and secreted by both neurons and glia (Kvajo et al., 2004; Giau et al., 2005). PN-1 expression is widely distributed throughout the adult mouse brain, with a few subpopulations of cells, including olfactory bulb glomeruli, neocortical layer V pyramidal neurons, and cells in the striatum, showing high expression levels (Mansuy et al., 1993). To elucidate serpin-protease interactions and their effects on brain function, several PN-1 transgenic and knockout mouse lines have been generated (Lüthi et al., 1997; Kvajo et al., 2004).

Lüthi et al. generated PN-1 knockout mice and transgenic mice overexpressing PN-1 under the control of the Thy 1 promoter (Thy 1/PN-1) to investigate the effects of disturbing the balance between PN-1 and its target proteases (Lüthi et al., 1997). Hippocampal slice electrophysiology recordings from both mouse lines yielded interesting results. While neither overexpression nor loss of PN-1 affected basal synaptic transmission or short-term synaptic plasticity, each manipulation resulted in increased polyspiking after repetitive afferent stimulation, which corresponded to lowered seizure thresholds after kainate injection. LTP induced by theta-burst stimulation revealed enhanced LTP in the Thy 1/PN-1 overexpressing mice, but decreased LTP in the PN-1 knockout mice. Whole-cell patch-clamp recordings in CA1 pyramidal cells to investigate mechanisms contributing to these effects revealed no differences in AMPAR/NMDAR ratios but did find prolonged NMDAR-mediated excitatory postsynaptic current (EPSC) decay times in the Thy 1/PN-1 mice. PN-1 knockout mice displayed markedly reduced AMPAR/NMDAR ratios but normal NMDAR-mediated EPSC decay times. The observed effects on NMDAR-mediated EPSC decay times and AMPAR/NMDAR ratios provide explanations for the changes in LTP magnitudes associated with increased or lost PN-1 activity; however, how both genetic manipulations can result in similar increases in epileptic activity is not clear (Lüthi et al., 1997).

The interaction between PN-1 activity and NMDAR function was further explored using a mouse line generated by inserting a β -galactosidase reporter gene following the PN-1 coding sequence (Kvajo et al., 2004). PN-1 reporter and knockout mice were subjected to a sensory deprivation model in which all the whiskers on one side except for the D1 whisker were trimmed and biochemical and electrophysiological changes in the barrel cortex were observed after whisker-dependent exploration in an enriched environment. In the PN-1 reporter mice, these studies revealed increased PN-1 activity in the barrel cortex and cortical layers II/III after 24 hours of whisker-dependent exploration, which spread to cortical layers IV and V and several thalamic nuclei receiving projections after 72 hours of whisker-dependent exploration. Because tPA is a target of PN-1, tPA activity after single-whisker exploration was examined. In the PN-1 reporter mice, tPA was upregulated in the same layer II/III and V cells in which PN-1 was upregulated. The PN-1 knockout mice, however, showed significantly increased tPA activity throughout somatosensory cortex and the hippocampus. This increased tPA activity was further associated with marked decreases in

GluN1 immunoreactivity, suggesting increased cleavage of NMDAR receptors resulting in increased removal of NMDARs from the cell surface, or increased NMDAR regulation via low-density lipoprotein receptors. Electrophysiological recordings in the barrel cortex demonstrated that PN-1 knockout mice had significantly reduced NMDAR-mediated currents and AMPAR/NMDAR ratios, and also showed significantly reduced sensory-evoked potentials (Kvajo et al., 2004).

Recently, both the PN-1 reporter and knockout mice were used to assess PN-1 function in cued fear conditioning and fear extinction (Meins et al., 2010). These experiments demonstrated that PN-1 is widely expressed throughout the amygdala, primarily by inhibitory neurons and glial cells. While PN-1 wild-type and knockout mice showed similar freezing levels in cued fear conditioning, PN-1 knockout mice displayed significant deficits in fear extinction, as evidenced by persistently higher freezing levels with repeated exposures to the conditioned stimulus. The impaired fear extinction in the PN-1 knockouts was further associated with altered expression levels of Fos and α -calcium/calmodulin protein kinase II (α CamKII) phosphorylation across different amygdala nuclei (Meins et al., 2010).

3.2. Neuroserpin

Neuroserpin, another serpin highly expressed in the brain, strongly inhibits tPA but has no inhibitory action against plasmin and thrombin (Krueger et al., 1997; Hastings et al., 1997; Barker-Carlson et al., 2002; Miranda and Lomas, 2006). In the adult mouse brain, neuroserpin is highly expressed in the neocortex, olfactory bulbs, hippocampus, amygdala, and cerebellum (Krueger et al., 1997). Like many of the serine proteases and serpins discussed thus far, neuroserpin is synthesized and secreted by both neurons and glia (Krueger et al., 1997; Hastings et al., 1997; Docagne et al., 1999). The expression patterns of neuroserpin and its primary target, tPA, highly overlap, suggesting a tight functional relationship between these two enzymes (as reviewed in Yepes and Lawrence, 2004a; Yepes and Lawrence, 2004b; and Miranda and Lomas, 2006).

While tPA is the frontline treatment for embolic or thrombotic stroke because of its strong thrombolytic activity, there is much debate surrounding tPA's effects on neurons (Gingrich and Traynelis, 2000; Yepes and Lawrence, 2004a; Yepes and Lawrence, 2004b; Echeverry et al., 2010). Thus, insights into neuroserpin's roles in synaptic function have come mainly from work done in models of neuronal injury. Like tPA, neuroserpin is rapidly released and its transcription is upregulated in cultured hippocampal neurons following neuronal depolarization, implying that neuroserpin activity can be regulated in an activity-dependent manner (Berger et al., 1999; Samson and Medcalf, 2006). Administration of exogenous neuroserpin, by either addition to media for cultured cortical neurons, or direct injection into striatum or cortex is neuroprotective against NMDA-induced excitotoxicity (Lebeurrier et al., 2005). Neuroserpin is also neuroprotective against excitotoxicity in ischemic stroke and seizure models, by both tPA inhibition-dependent and tPA inhibition-independent mechanisms (Cinelli et al., 2001; Wu et al., 2010; Yepes et al., 2002).

The role of neuroserpin in normal brain function has been investigated using mice overexpressing or lacking neuroserpin (Madani et al., 2003). Interestingly, both manipulations resulted in similar behavioral phenotypes: impaired exploratory behavior, increased anxiety-like behavior, and reduced interaction with a novel object (Madani et al., 2003). A possible mechanism underlying these behavioral deficits was proposed by recent observations that neuroserpin overexpressed in cultured hippocampal neurons resulted in increased density of dendritic protrusions and shifted spine morphology to a more immature phenotype (Borges et al., 2010). Taken together with observations that neuroserpin overexpressing mice show markedly reduced tPA activity (Cinelli et al., 2001), and that

stress-induced changes in CA1 pyramidal cell spine morphology follow upregulation of tPA activity (Pawlak et al., 2003; Pawlak et al., 2005b), this mechanism is consistent with the idea that neuroserpin tightly regulates tPA activity. However, the observation that neuroserpin-deficient mice did not display dysregulated tPA activity (Madani et al., 2003; Wu et al., 2010) suggests that neuroserpin can influence behavior via a mechanism independent of inhibiting tPA activity.

The profound effects of mutated neuroserpin function in the CNS are underscored by the discovery of a neurodegenerative disease caused by point mutations in neuroserpin, *familial encephalopathy with neuroserpin inclusion bodies* (FENIB; Davis et al., 1999a). Affected individuals present with a constellation of symptoms, which include progressive dementia, tremor, and epilepsy. The pathological hallmark of FENIB, however, is the presence of inclusion bodies in neurons, called Collins bodies, comprised of polymerized mutant neuroserpin (Davis et al., 1999a; Molinari et al., 2003; Miranda and Lomas, 2006). In postmortem tissue, the presence of Collins bodies was most prominent in the deeper layers of the cortex, the insular cortex, the cingulate gyrus, and the substantia nigra (Davis et al., 1999b). Four different point mutations in neuroserpin that all result in FENIB have been identified thus far, and the location of the mutation can affect the age of onset and the severity of the disease (Davis et al., 2002). These mutations are clustered in the —shutter region of neuroserpin, which in normal function is critical for promoting the stability of the serpin-inactivated serine protease complex, but, in FENIB, promote the polymerization of mutant neuroserpin (Briand et al., 2001; Davis et al., 2002; Molinari et al., 2003; Miranda and Lomas, 2006; Ricagno et al., 2009). While it is clear that mutant neuroserpin can disrupt normal brain function, it is still unclear how these neuroserpin aggregates exert their deleterious effects.

Recent reports suggest that neuroserpin could also influence neuropathology associated with Alzheimer's disease; however, both neuroprotective and deleterious effects have been observed. Neuroserpin has been shown to be highly colocalized with amyloid plaques in brain tissue from Alzheimer's disease patients and, *in vitro*, can bind A β _{1–42} peptide fragments in a 1:1 stoichiometry. Interestingly, A β _{1–42} in complex with neuroserpin can still form aggregates, however, these aggregates were small and amorphous, rather than the fibrillar configuration typically seen in the disease. Furthermore, these atypical aggregates were not toxic to cultured cortical neurons, suggesting that neuroserpin could be neuroprotective in Alzheimer's disease (Kinghorn et al., 2006). This idea is challenged, though, by observations that high concentrations of neuroserpin are correlated with elevated levels of total tau protein in cerebrospinal fluid from probable Alzheimer's disease patients (Nielsen et al., 2007), and that inhibition of plasminogen activator activity concomitant with upregulated neuroserpin could potentially lead to impaired A β clearance in postmortem Alzheimer's disease brains (Fabbro and Seeds, 2009). Collectively, these results suggest that the mechanisms underlying neuroserpin's actions are complex, and that serine protease-serpin interactions warrant further investigation.

4. Protease-activated receptors

Protease-activated receptors (PARs) comprise a unique family of four GPCRs (PAR1–4) that are activated by proteolytic cleavage of their amino terminus by serine proteases such as thrombin, plasmin and trypsin (Fig. 2A). This cleavage reveals a new amino terminus that acts as a tethered ligand to activate the receptor (Fig 2B). Once activated, the PARs have been shown to couple to multiple G α proteins and their associated signaling cascades; thus, activation of PARs can have pleiotropic effects. In addition to the serine proteases highlighted above, the PARs can also be activated by short peptides sequences mimicking the tethered ligand. These short agonist peptides have become invaluable tools for

investigating the roles of PARs in the many cell types in which they have been found. Table 2 lists tethered ligands, known agonist peptides, and known activating serine proteases in the CNS for the four members of the PAR family. General properties of the PARs—structure, activation, and signaling—have been extensively reviewed (Coughlin, 2000; O'Brien et al., 2001; Vergnolle et al., 2001; Macfarlane et al., 2001; Hollenberg and Compton, 2002; Traynelis and Trejo, 2007; Ramachandran and Hollenberg, 2008). A few recent reviews have also discussed PARs in various aspects of CNS development, neuroprotection, and neurodegeneration (Noorbakhsh et al., 2003; Rohatgi et al., 2004b; Luo et al., 2007; Sokolova and Reiser, 2008). This section will address our current understanding of PAR function in learning and memory and synaptic plasticity.

4.1. PAR1

The most widely studied member of this intriguing GPCR family is PAR1. Experiments performed in many different cell types have found that PAR1 can couple to *Gaq/11*, *Gai/o*, *Gα12/13*, and their respective intracellular signaling pathways (as discussed in Coughlin 2000, Macfarlane et al., 2001; and Ramachandran and Hollenberg, 2008). In the CNS, PAR1 activation has a number of effects, ranging from upregulated PAR1 expression, nerve growth factor (NGF) secretion, neurite retraction, and astrocyte proliferation (Noorbakhsh et al., 2003). In mammalian brains, PAR1 expression has been found in specific neuronal and glial populations in the cortex, the basal ganglia, the striatum, and the nucleus accumbens (Weinstein et al., 1995; Niclou et al., 1998; Striggow et al., 2001; Balcaitis et al., 2003; Junge et al., 2004). PAR1 expression is also high in the amygdala and the hippocampus, important brain areas for learning and memory. In both human and rodent hippocampus, evidence from several studies suggests that PAR1 is expressed primarily in astrocytes (Weinstein et al., 1995; Striggow et al., 2001; Junge et al., 2004; Gomez-Gonzalo et al., 2010).

Although endogenous activators for PAR1 in the CNS have not yet been defined, the mRNA for the thrombin precursor, prothrombin, and the plasmin precursor, plasminogen, are both expressed in the brain (Weinstein et al., 1995; Qian et al., 1993; Tsirka et al., 1995), and the tPA/plasmin signaling system described above is a viable candidate as a PAR1 regulating system (Junge et al., 2003; Lee et al., 2007). As may be expected from the previous discussions of thrombin, tPA, and plasmin, PAR1 has been extensively studied for its roles in neuronal damage after ischemic or traumatic injury. In ischemia and hypoxia models, ablation or pharmacological blockade of PAR1 resulted in reduced infarct volume (Junge et al., 2003; Olson et al., 2004). In a cortical stab wound model, PAR1 activation triggered astrogliosis associated with glial scar formation after traumatic brain injury (Nicole et al., 2005). PAR1 inhibition or deletion was also neuroprotective in dopaminergic nerve terminals in the striatum in a mouse model of Parkinson's disease (Hamill et al., 2007).

Accumulating lines of evidence are beginning to reveal diverse roles for PAR1 in normal brain function. In nicotine and morphine dependence models, activation of PAR1 on dopaminergic neurons regulated nicotine-induced dopamine release and conditioned place preference and hyperlocomotion (Nagai et al., 2006; Ito et al., 2007). PAR1 activation has also been shown to modulate synaptic responses in the hippocampus. In whole-cell recordings from CA1 pyramidal cells, PAR1 activation was demonstrated to potentiate NMDAR-mediated currents (Gingrich et al., 2000; Lee et al., 2007). Moreover, this enhanced NMDAR response facilitated the induction of LTP in hippocampal extracellular field recordings (Maggio et al., 2008). Collectively, these observations suggest that PAR1 activity may influence NMDAR-dependent memory formation and synaptic plasticity.

Indeed, using PAR1 knockout mice (Connolly et al., 1996), our laboratory has demonstrated that loss of PAR1 leads to learning and memory deficits in passive avoidance, cued fear

conditioning, and contextual fear conditioning tasks (Almonte et al., 2007; Almonte et al., in preparation). A critical importance for intact PAR1 function was revealed in LTP experiments showing that slices from PAR1 knockout mice have strikingly decreased levels of theta-burst induced LTP (Almonte et al., 2009; Almonte et al., in preparation). Interestingly, loss of PAR1 results in an insurmountable diminution in the maximal level of potentiation attainable with repeated theta-burst stimulations. In addition to demonstrating that PAR1 knockout mice have no deficits in normal baseline synaptic transmission or short term plasticity, we found that neither NMDAR-mediated fEPSP responses nor expression levels of AMPAR and NMDAR subunits are impaired (Almonte et al., in preparation). Coupled with the previous observation that NMDA-evoked current responses are normal in PAR1 knockout mice (Gingrich et al., 2000), these lines of evidence suggest that PAR1 is a novel mediator of neuronal activity-dependent plasticity.

4.2. PAR2

PAR2 is widely expressed in the brain, particularly in the hippocampus, amygdala, and cortex, and is found in neurons and glia (Smith-Swintosky et al., 1997; Striggow et al., 2001; Bushell et al., 2006; Bushell, 2007; Lohman et al., 2008). Upon activation, PAR2 has been shown to couple to *Gaq/11* and *Gai/o* (Sorensen et al., 2003; Ramachandran and Hollenberg, 2008; McCoy et al., 2010). While little is known about PAR2 function in the brain, recent studies have begun to address the contribution of PAR2 activity to synaptic function and behavior. As with PAR1, the endogenous activators for PAR2 in the brain are not known, but a couple of candidates are the trypsin-like serine proteases, mesotrypsin and neuropsin, discussed in a previous section. In contrast to the PAR1 ischemia studies, PAR2 knockout mice display significantly increased infarct volumes in a transient ischemia/reperfusion model, suggesting a neuroprotective role for PAR2 function (Jin et al., 2005). Consistent with this observation are findings that PAR2 activation was neuroprotective against seizures in an *in vivo* electrical amygdala-kindling seizure model (Lohman et al., 2008) and in an *in vitro* model of kainite-induced neurotoxicity (Greenwood and Bushell, 2010). Interestingly, systemic injection of a PAR2 activating peptide in a rat strain showing high baseline levels of anxiety resulted in impaired performance in the test-retest version of the elevated plus maze and in the Morris water maze (Lohman et al., 2009). A possible explanation for these impairments comes from slice electrophysiology experiments demonstrating that application of a PAR2 activating peptide could modulate both neuronal excitability and synaptic transmission (Gan et al., 2009). Taken together, these observations suggest a fine balance between beneficial and untoward effects of PAR2 activity.

4.3. PAR3 and PAR4

While several studies have shown PAR3 and PAR4 expression in the brain, there is a dearth of information on their effects on synaptic function and behavior (see Striggow et al., 2001; Luo et al., 2007; and Sokolova and Reiser, 2008 for further discussions). Studies in other cell types have shown that thrombin can cleave PAR3 at low concentrations and PAR4 at high concentrations. Curiously, PAR3 does not seem to signal by itself, as peptides corresponding to the predicted tethered ligand sequence fail to elicit responses when PAR3 is expressed alone. However, when coexpressed with PAR4, PAR3 forms heterodimers with PAR4 and seems to act as a cofactor for PAR4 activation and signaling through *Gaq/11* coupling at low thrombin concentrations (as reviewed in Coughlin, 2000; Macfarlane et al., 2001; and Ramachandran and Hollenberg, 2008). Reminiscent of the studies of PAR1, PAR4 expression was highly upregulated in both neurons and glia in ischemia models (Rohatgi et al., 2004a; Henrich-Noack et al., 2006; Henrich-Noack et al., 2010). Moreover, reduced infarct volumes were recently observed in PAR4 knockout mice in a transient ischemia/reperfusion model (Mao et al., 2010).

5. Emerging role for PARs in astrocyte-neuron interactions

A growing body of literature suggests an exciting role for PARs in astrocyte-neuron interactions. Several studies show that in some brain regions, such as the hippocampus (Weinstein et al., 1995; Striggow et al., 2001; Junge et al., 2004), the entorhinal cortex (Gomez-Gonzalo et al., 2010), and the nucleus of the solitary tract (Hermann et al., 2009), PAR1 is preferentially expressed in astrocytes. Activation of PAR1 in astrocytes leads to the vesicular release of glutamate, which can be sensed by NMDARs on neurons, and can thus potentiate NMDAR function (Gingrich et al., 2000; Lee et al., 2007; Mannaioni et al., 2008; Shigetomi et al., 2008). A recent study also indicates that PAR2-mediated release of transmitters and cytokines from astrocytes is neuroprotective against kainite-induced neurotoxicity. (Greenwood and Bushell, 2010). Thus, PAR1 and PAR2 may elicit their observed effects on synaptic plasticity and behavior in part through mediating processes underlying the function of the —tripartite synapse (Haydon and Carmignoto, 2006; Fellin et al., 2009; but also see Agulhon et al., 2008; Fiacco et al., 2009; Agulhon et al., 2010; Wenker, 2010). In Fig. 3, we propose a model in which activation of astrocytic PAR1 by the tPA/plasminogen/plasmin system or thrombin leads to Gαq-coupled GPCR-Ca²⁺ signaling-dependent vesicular release of glutamate that activates NMDARs in postsynaptic neurons, which, in turn, engages molecules important for synaptic plasticity and memory formation (Sweatt, 2004; Day and Sweatt, 2010).

Our proposed model also highlights some current gaps in our understanding of PAR1 function. First, we do not have a full grasp on the effects of neurotransmitters that may be released by astrocytes following PAR1 activation. It is possible that, in addition to activating NMDARs, released glutamate could activate metabotropic glutamate receptors (mGluRs). Activation of astrocytic PAR1 has also been shown to result in the release of ATP and UDP-glucose that can activate purinergic receptors (e.g., P2X or P2Y receptors; Kreda et al., 2008; Blum et al., 2008). Additionally, other neuroactive molecules, such as D-serine (Mothet et al., 2005; Henneberger et al., 2010) and tumor-necrosis factor-α (TNF-α; Stellwagen and Malenka, 2006), could also be released following PAR1 activation to modulate synaptic activity.

Second, the idea of exocytotic release of transmitter from astrocytes is highly controversial, and other release mechanisms, such as channel-mediated release, have been suggested (Fiacco et al., 2009; Hamilton and Attwell, 2010). Interestingly, activation of astrocytic PAR1 can also lead to the activation of the calcium-activated anion channel Bestrophin-1, which is permeable to Cl[−], bicarbonate, and large anions such as glutamate (Park et al., 2009; Lee et al., 2010; Park et al., submitted). Glutamate released through Bestrophin-1 following PAR1 activation activates neuronal NMDARs, and this glutamate release can be eliminated by silencing Bestrophin-1 expression using shRNA. Furthermore, a subthreshold LTP induction stimulus combined with application of a PAR1 agonist resulted in LTP, which was blocked by an NMDAR antagonist and by inhibition of Bestrophin-1 with an anion channel blocker and gene silencing with the Bestrophin-1 shRNA (Park et al., submitted). Thus, PAR1 activation can elicit astrocytic neurotransmitter release via multiple release mechanisms to affect synaptic function (Lee et al., 2007; Mannaioni et al., 2008; Shigetomi et al., Lee et al., 2010; Park et al., submitted).

Next, in brain regions where PAR1 is expressed in neurons, PAR1 could mimic other GPCRs in regulating NMDARs through activation of Src family kinases (Salter and Kalia, 2004). Finally, PAR1 in neurons could potentially influence synaptic function and plasticity by inducing cytoskeletal changes via its coupling to Gα12/13 (Gill et al., 1998; Majumdar et al., 1999; Rex et al., 2010).

6. Summary

It is becoming increasingly clear that the interplay of serine proteases, their serpin inhibitors, and their target protease-activated receptors compose a triad that can regulate many inter- and intra-cellular mechanisms subserving behavior and synaptic plasticity (Fig. 4). Aberrant activity of one or more components of this triad can lead to deleterious effects, including mental retardation, increased susceptibility to seizures, and neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease. Thus, a better understanding of the intricate balance of activities within this triad could lead to the development of novel therapeutics to treat these disorders. To achieve this goal, however, a few uncertainties will need to be addressed in future studies. First, the full repertoire of serine proteases and serpins endogenously expressed in the brain is not known. Second, as many *in vitro* studies have illustrated, serine proteases can cleave multiple PAR and non-PAR substrates, serpins can inhibit multiple proteases, and PARs can signal through multiple G α proteins, thus making investigating even a single component of this triad a challenging endeavor. Lastly, serine protease, serpin, and PAR expression have been found in both neurons and glia, leading to interesting questions concerning glial-neuronal signaling via this system. The development of more refined pharmacological tools and cell-type specific inducible transgenic mice to address these uncertainties is eagerly awaited.

Acknowledgments

We thank Stephen Traynelis and Manuel Yepes for helpful comments on the review. We also thank Justin Lee for kindly sharing data and Laura Qadri for help in generating figures. This work was supported by NIH grants MH57014, AG031722, NS 057098, and funds from the Evelyn F. McKnight Brain Research Foundation.

List of abbreviations

AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
ERK/MAPK	extracellular signal-regulated kinase/mitogen-activated protein kinase
EPSC	excitatory postsynaptic current
fEPSP	field excitatory postsynaptic potential
GPCR	G-protein coupled receptor
IPSC	inhibitory postsynaptic current
LTP	long-term potentiation
NMDAR	N-methyl-D-aspartate receptor
PAR	protease-activated receptor
tPA	tissue plasminogen activator

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Figure 1. Proteolytic actions of the tPA/plasmin system

The primary target of tPA is the proenzyme plasminogen, which it cleaves into the active serine protease, plasmin. Additionally, tPA has other proteolytic targets, such as the GluN1 subunit of the NMDAR, and non-proteolytic effects, such as its interactions with the GluN2B NMDAR subunit, and with the low-density lipoprotein receptor (LDLR). Plasmin, a broad spectrum protease, has a variety of proteolytic targets, such as PAR1, the proenzyme proBDNF, and the amino-terminal domain (ATD) of the GluN2A NMDAR subunit.

Figure 2. Tethered ligand mode of activation of protease-activated receptors

(A) Protease-activated receptors (e.g., PAR1) are activated by proteolytic cleavage of their amino terminus by serine proteases, such as thrombin or plasmin. (B) A new amino terminus is unmasked (shown in orange), which then acts as a tethered ligand to activate the receptor and initiate intracellular signaling cascades.

Figure 3. PAR1 is a potential model for astrocyte-neuron interactions in the hippocampus
 Activation of astrocytic PAR1 by the tPA/plasminogen/plasmin system or thrombin leads to $G_{\alpha q}$ -coupled GPCR- Ca^{2+} signaling-dependent vesicular release of glutamate that activates NMDARs in postsynaptic neurons. Synaptic NMDAR activation leads to Ca^{2+} influx and the engagement of a number of signal transduction proteins necessary for memory formation and synaptic plasticity, such as CaMKII, PKC, Pyk2, and ERK. These molecules, in turn, activate nuclear proteins that can regulate gene transcription, such as the transcription factors CREB and Elk-1 and epigenetic regulators, such as histone acetyltransferases (HATs), histone deacetylases (HDACs), and DNA methyltransferases (DNMTs). However, it is possible that, in addition to activating NMDARs, released glutamate could activate metabotropic glutamate receptors (mGluRs). Activation of astrocytic PAR1 has also been shown to result in the release of ATP that can activate P2X purinergic receptors. Additionally, in brain regions where PAR1 is expressed in neurons, PAR1 could mimic other GPCRs in regulating NMDARs through activation of Src family kinases.

Figure 4. Serine proteases, serpins, and PARs form a triad that can regulate synaptic plasticity and memory formation

Various serine proteases, through either proteolytic activity or through PAR activation, have been demonstrated to influence synaptic plasticity and memory formation. Serpins function primarily to inhibit the activities of serine proteases, but have also been shown to regulate synaptic plasticity and memory formation. Direct effects on PAR activity by serpins are as yet unknown. In addition, proteolytic-independent effects of serine proteases, and inhibitory-independent effects of serpins on synaptic plasticity and memory formation are not yet known.

Table 1

Serine proteases implicated in synaptic plasticity and memory formation and their (known) inhibitors in the brain.

Serine protease	Serine protease inhibitor
Thrombin	Protease-nexin-1 (PN-1), antithrombin III colligin, phosphatidylethanolamine-binding protein
Tissue plasminogen activator (tPA)	Plasminogen activator inhibitor-1 (PAI-1), neuroserpin, PN-1
Plasmin	α_2 -antiplasmin, PN-1
Trypsin	PN-1, α_1 -antitrypsin
Neuropsin	Serine protease inhibitor 3, murinoglobulin I

Table 2

Protease-activated receptors, agonist peptides, and activating proteases in the brain.

	PAR1	PAR2	PAR3	PAR4
Size	425 aa	397 aa	374 aa	385 aa
Tethered ligand sequence	SFLLR (human, rat, mouse)	SLIGKV (human) SLIGRL (rat, mouse)	TFRGAP(human) SFNGGP(mouse)	GYPGQV(human) GFPGKP (rat) GYPGKF(mouse)
Agonist peptides	SFLLR-NH ₂ TFLLR-NH ₂	SLIGKV-NH ₂ SLIGRL-NH ₂ SFLLRN-NH ₂ <i>trans</i> -cinnamoyl- LIGRLO-NH ₂	None ^a	GYPGQV-NH ₂ GFPGKP-NH ₂ GYPGKF-NH ₂ AYPGKF-NH ₂
Activating proteases	Thrombin, plasmin, trypsin, factor VIIa, factor Xa, activated protein C, cathepsin G, granzyme A, MMP-1, kallikrein-6	Trypsin, mast cell tryptase, tissue factor/ factor VIIa, factor Xa, membrane-type serine protease 1, kallikrein-5, -6, -14,	Thrombin, trypsin	Thrombin, trypsin, cathepsin G

^aPeptides corresponding to the tethered ligand sequence do not activate PAR3.